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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goals of this research project is to characterize how disruptor peptide blocks beta-catenin interaction with PTH receptor, inhibits chondrocyte hypertrophy and prevents osteoarthritis (OA) progression. In the first year, we completed the most work in the Aim 1 and initiated some work in Aim 2. We designed a disruptor peptide corresponding to the carboxyl-terminal region of PTH receptor, and found this disruptor peptide inhibited beta-catenin binding to PTH receptor by GST-pull down assay. We also confirmed that disruptor peptide conjugated to penetratin can enter cells. Importantly, disruptor peptide can reverse the beta-catenin-mediated PTH receptor signaling switch by increasing Gs/cAMP signaling and inhibiting Gq/PLC activation in chondrocytes. In addition, we successfully induced ATDC5 cell differentiation from proliferating chondrocytes to the hypertrophic stage, and generated mouse OA model surgically induced by destabilization of the medial meniscus. These results provide the foundation for further studies whether disruptor peptide can inhibit chondrocyte hypertrophy in vitro and protect cartilage damage in a mouse OA model.

15. SUBJECT TERMS

Osteoarthritis**;** Parathyroid hormone-related protein; PTH receptor; Beta-catenin; Chondrocyte hypertrophy

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1. INTRODUCTION:

Chondrocyte hypertrophy has been established to be involved in the pathogenesis of osteoarthritis (OA). The application of a disruptor peptide to interfere with protein-protein interaction represents a novel therapeutic strategy for inhibition of chondrocyte hypertrophy and treatment/prevention of OA. The purpose of our proposal is to design and develop a novel disruptor peptide and test its efficacy in relevant cellular and in vivo OA models, thus accomplishing most pre-clinical goals necessary for ultimate human subject testing.

2. KEYWORDS:

Osteoarthritis

Parathyroid hormone-related protein

PTH receptor

β-catenin

Cell signaling

Chondrocyte hypertrophy

Destabilization of the medial meniscus

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The goals of this research plan is to characterize how disruptor peptide blocks beta-catenin interaction with PTH receptor, inhibits chondrocyte hypertrophy and prevents OA progression.

What was accomplished under these goals?

My grant project entitled “Inhibition of chondrocyte hypertrophy of osteoarthritis by disruptor peptide” started from July 1, 2016 and will end on December 31, 2017. During the first year of my research, we completed the most work in Aim 1 and initiated Aim 2 studies according to the time line in my Discovery Award.

1. Major activities.

The application of disruptor peptide to target the β-catenin interaction with PTH receptor represents a novel therapeutic strategy for the inhibition of chondrocyte hypertrophy and treatment/prevention of OA.

2. Specific objectives

Aim 1 will develop and characterize a disruptor peptide specifically blocks the interaction of beta-catenin with PTHR and inhibits the pathogenic beta-catenin-mediated PTHR signaling switch. In Aim 2, we will define the role of disruptor peptide in inhibiting chondrocyte differentiation in a mouse DMM (destabilization of the medial meniscus) model.

3. Significant results

a. Design a disruptor peptide corresponding to the carboxyl-terminal region of PTH receptor (PTHR).

Previous findings show that deletion of 5 and 10 amino acids from the carboxyl-terminus of PTHR residues (589–593 and 584–593) abolished the binding of β-catenin to PTHR, whereas a deletion of up to the last 4 amino acids in the carboxyl-tail (ETVM), which are PDZ protein NHERF1 and 2 binding sites, did not affect this binding. Mutagenesis analysis of this region identified the critical binding sites of the β-catenin protein to be W589, L585, and L584 of PTHR. The last 10 amino acids in the carboxyl-tail of human PTHR are identical to those in rat/mouse PTHR. Therefore, we propose that the carboxyl-terminal region of 6 amino acids is the β-catenin recognition motif in PTHR. These disruptor peptide were synthesized by PEPTIDE 2.0 (Chantilly, VA).

b. Inhibition of β-catenin binding to PTHR by GST-pull down assay by disruptor peptide

We generated His-tagged β-catenin and GST-tagged PTHR proteins. His-β-catenin protein (500 nM) and 500 nM GST-PTHR were incubated in the presence of different concentrations (0~100 μM) of carboxyl-terminal six-amino-acid disruptor peptide Dis-pep) or its control peptide (Ctr-pep), which does not interact with β-catenin, in pull-down buffer. The GST-pull down data showed that the disruptor peptide concentration-dependently inhibited β-catenin binding to PTHR (**Figure 1**).

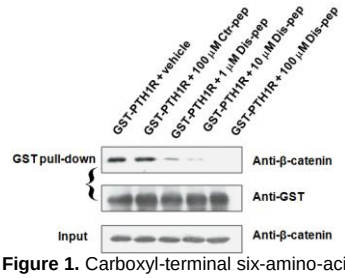

Figure 1. Carboxyl-terminal six-amino-acid disruptor peptide (Dis-pep) but not its control peptide inhibits the interaction of PTHR with β-catenin.

c. Disruptor peptide enters cells.

Cellular delivery of peptides is facilitated by peptide conjugation with the carrier, cell-penetrating peptides. To assess disruptor peptide permeability into cells, the effects of disruptor peptide conjugated to penetratin (Pen), which can transport peptide into live cells and animals. To confirm the peptide directly enters cells, we generated the disruptor peptides conjugated with green fluorescent protein (GFP) and/or Pen. Primary chondrocytes were treated with 10 μM of disruptor peptides conjugated with GFP and/or Pen for 2h. The peptides in culture medium were then fully washed with PBS and live cells were visualized using confocal microscopy. Disruptor peptide conjugated with GFP and Pen successfully entered cells (**Figure 2)**.

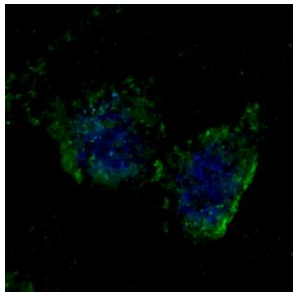

Figure 2. GFP tagged Pen-6-cdp enters chondrocytes. Nuclear staining was shown in blue.

d. Disruptor peptide reverses the β-catenin-mediated PTHR signaling switch in chondrocytes.

Mouse primary chondrocytes express both β-catenin and PTHR. Our data showed that Pen-dis-pep concentration-dependently increased 10 nM PTH-induced cAMP formation, while Pen-ctr-pep had no effect on PTH-stimulated cAMP formation. Pen-dis-pep itself had no effects on cAMP formation without PTH treatment. To evaluate PTHR-mediated Gαq/PLC signaling, intracellular calcium mobilization ($[Ca^{2+}]_i$) was measured. Pretreatment with Pen-dis-pep for 2 h concentration-dependently inhibited PTH (100 nM)-induced $[Ca^{2+}]_i$.

Collectively, these data demonstrate that the disruptor peptide limits the PTHR signaling switch to increase Gαs/cAMP activation.

e. Disruptor peptide has no effect on Wnt3a-induced β-catenin/ T-cell factor (TCF) activation.

Primary chondrocytes were transfected with TOPflash or FOPflash. 36h later, cells were pretreated with Pen-dis-pep (10 μM) for 2h, and then Wnt3a (50 ng/ml) were added for 8h. After treatment, the TOPflash and FOPflash were measured. Our data demonstrated disruptor peptide did not affect the canonical Wnt pathway. In addition, different concentrations of disruptor peptide (1~100 μM) did not cause any cytotoxicity as per MTT assay.

f. Differentiation of ATDC5 cells from proliferating chondrocytes to the hypertrophic stage.

Mouse ATDC5 cells undergo an orderly series of chondrogenic differentiation. The differentiation of ATDC5 cells was induced by addition of ITS (10 µg/ml insulin, 10 µg/ml transferrin, and 10 ng/ml sodium selenite) into the cell culture medium after cell confluence. The typical nodules were formed by day 30 after ITS treatment and measured by phase-contrast microscopy (**Figure 3a, b and c**) and the staining of alcian blue, a proteoglycan stain (**Figure 3d, e, and f)**. These data suggest ITS induced the transition of ATDC5 cells from proliferating chondrocytes to the hypertrophic stage.

g. Generation of mouse DMM model of OA.

The mouse OA model surgically induced by destabilization of the medial meniscus (DMM) has been commonly used and shares many features of human OA. Mouse DMM model was induced by transection of the meniscotibial ligament in right leg. After 8 weeks, we isolated right and left knee joints. Histological changes and analyses of disease severity were examined by Safranin O staining and Fast green staining, suggesting cartilage damages occurs in the DMM mouse model (**Figure 4**). We will investigate whether disruptor peptide protects against cartilage damage in this mouse OA model.

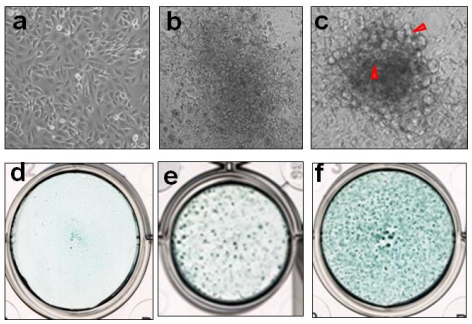

Figure 3. Differentiation of ATDC5 cells induced by ITS. In (a-c), cells were induced by ITS for 1, 14 and 30 days. Cartilage nodule on day 30 was shown in (c). Arrowheads indicate hypertrophic cells. Alcian blue staining was shown on days 1 (d), 14(e), and 30.

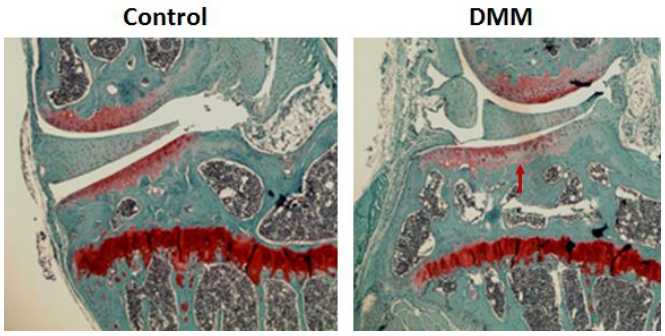

Figure 4. Histological change at 8 weeks following the surgery of destabilization of the medial meniscus. Slide were stained with Safranin-O and Fast green. Arrow indicates cartilage loss.

4. Other achievements

None

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

(1) Effect of disruptor peptide on PTHrP-induced PTHR coupling to Gα protein subunits

PTHrP-induced PTHR coupling to Gα protein subunits will be determined by [^{35}S]GTPγS binding and immunoprecipitation of [^{35}S]GTPγS-bound Gα subunits using specific anti-Gαs and Gαq antibody, respectively.

(2) Effects of disruptor peptide on the binding of PTHR to β-catenin in chondrocytes.

The experiments will be performed by immunoprecipitation assay. The chondrocytes will be treated in the presence or absence of disruptor peptide. The cells will be lysed and solubilized materials will be incubated with PTHR polyclonal antibody for 1 h at 4°C, and then protein A-Sepharose 4B conjugate will be added and incubated overnight at 4°C. Total lysates and immunoprecipitated proteins will be analyzed by SDS-polyacrylamide gels and band intensity for β-catenin and PTHR will be quantified using the Licor Odyssey system.

(3) Effect of disruptor peptide on inhibition of chondrogenic cell differentiation *in vitro*.

We will use chondrogenic medium containing ITS to induce ATDC5 cell differentiation in the presence or absence of PTHrP, disruptor peptide and control peptide conjugated with penetratin. The mRNA expression of type II collagen a1 (Col2a1), aggrecan, Col10a1 and matrix metalloproteinase 13 (MMP13) will be measured dynamically. To further verify our results, we will repeat the experiments with mouse bone marrow-derived mesenchymal stem cell differentiation to chondrocytes.

(4) Effect of disruptor peptide on protection of cartilage damage in a mouse OA model.

Two independent experiments will be performed. Experiment 1 is a feasibility experiment. Experiment 2 will be performed after the protective effects of disruptor peptide on cartilage damage in DMM model are evidenced in experiment 1. After termination of treatments, whole right and left knee joints from half of the mice in each group will be harvested separately. Histological changes and analyses of disease severity will be examined by hematoxylin & eosin (H&E) and Safranin O staining using standard protocols. The knee cartilage lesions will be analyzed. The markers of cartilage hypertrophy, such as, Col10a1 and MMP13, will be determined by immunohistochemical analysis. To achieve the cellular resolution of chondrocytes and quantitative assessment of articular cartilage volume and surface area, the knee joints from other half of mice in each group will be dissected and used for phase-contrast microCT. All the data will be analyzed for protective effects of disruptor peptide on cartilage damage.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Nothing to Report

6. PRODUCTS:

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Bin Wang, Ph.D., Principal Investigator (4.8 calendar months), Assistant Professor of Medicine, Center for Translational Medicine, Department of Medicine. As PI, Dr. Wang is responsible for all experiments outlined in the specific aims of the project including the design, performance, analysis, interpretation of experimental studies, preparation of manuscripts, and reporting results of the work.

Irving M Shapiro, BDS, PhD, Other Significant Contributor (no effort requested). Dr. Shapiro is an internationally-recognized cartilage biologist and Anthony and Gertrude DePalma Professor of Orthopaedic Surgery, Director, Division of Orthopaedic Research, Department of Orthopaedic Surgery. He discusses with Dr. Wang about data interpretation, and any design/troubleshooting issues.

Research Technician, Yanmei Yang (12 calendar months), is responsible for routine cell culture, preparing media and buffers for general experiment usage. She performs biochemical experiments, including the cAMP assay, Western blotting and real time PCR, and assist in all other experiments performed by Dr. Wang, including animal studies.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

9. APPENDICES:

Nothing to Report